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Food Chemistry

Straightforward rapid spectrophotometric quantification of total cyanogenic glycosides in fresh and processed cassava products

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ABSTRACT

In this study, we extend pioneering studies and demonstrate straightforward applicability of the corrin-based chemosensor, aquacyanocobyrinic acid (ACCA), for the instantaneous detection and rapid quantification of endogenous cyanide in fresh and processed cassava roots. Hydrolytically liberated endogenous cyanide from cyanogenic glycosides (CNp) reacts with ACCA to form dicyanocobyrinic acid (DCCA), accompanied by a change of colour from orange to violet. The method was successfully tested on various cassava samples containing between 6 and 200 mg equiv. HCN/kg as verified with isonicotinate/1,3-dimethylbarbiturate as an independent method. The affinity of ACCA sensor to cyanide is high, coordination occurs fast and the colorimetric response can therefore be instantaneously monitored with spectrophotometric methods. Direct applications of the sensor without need of extensive and laborious extraction processes are demonstrated in water-extracted samples, in acid-extracted samples, and directly on juice drops. ACCA showed high precision with a standard deviation (STDV) between 0.03 and 0.06 and high accuracy (93–96%). Overall, the ACCA procedure is straightforward, safe and easily performed. In a proof-of-concept study, rapid screening of ten samples within 20 min has been tested.

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1. Introduction

Cassava (*Manihot esculenta* Crantz) is a staple food in most tropical regions, and is grown over a range of climates and altitudes and on a wide variety of soils. The estimate total world cassava production in 2012 was 256 million tonnes according to FAO (2013), which is an increase of 40% since 2000. Africa represents the continent with the largest cassava production of more than 50% of the annual world production. The crop is one of the most important staple food with about 93% of the production used for human consumption (Nweke, Spencer, & Lynam, 2002). The roots of this important carbohydrate source are eaten both fresh and as processed products (Westby, 2002). However, in its tissue, cassava contains cyanogenic glycosides, mainly linamarin, that are enzymatically hydrolysed to glucose, acetone and hydrogen cyanide during cell rupture (Conn, 1994). The released cyanide is highly toxic for humans and is a threat to the cassava consumer (Rosling, 1988). The presence of cyanogenic glycosides in cassava tissues is related to illnesses that occur in populations where

cassava is the staple food. These illnesses include tropical ataxic neuropathy, epidemic spastic paraparesis, also known as *konzo* (Cliff, Muquingue, Nhassico, Nzwalo, & Bradbury, 2011; Rosling, 1988). These problems have been reported in the Democratic Republic of Congo, Nigeria, Tanzania and Mozambique (Ciglenecki et al., 2011; Mlingi, Nkya, Tatala, Rashid, & Bradbury, 2011; Nhassico, Muquingue, Cliff, Cumbana, & Bradbury, 2008).

Monitoring of cyanogenic potential (CNp) of cassava is therefore of utmost importance due to the following reasons: determination of safeness of different cassava products, evaluation of the efficiency of different existing methods of cassava processing on the removal of cyanogenic glycosides and determination of level of CNp of released new cassava varieties in breeding programmes. Unfortunately, all existing methodologies for the determination of CNp have been shown to be dependent either on analytical equipment or on laborious and slow procedures.

Various methods are used to determine cyanogens in cassava. Probably the most common analysis involves three main steps: (i) extraction of cyanogens from cassava, (ii) hydrolysis of CNp to cyanide and (iii) analysis of the latter (Borges, Fukuda, & Caldas, 1993; Bradbury, Bradbury, & Egan, 1994; Cook, 1978). Extraction of cyanogens from the plant material is normally carried out using dilute acid such as 0.1 M phosphoric acid in order to stop

endogenous linamarase activity and to stabilise the cyanohydrins (Cooke, 1978; Essers, Bosvel, van der Grift, & Voragen, 1993). Cyanogens can be hydrolysed to cyanide by acid hydrolysis (Bradbury, Egan, & Lynch, 1991), by autolysis (AOAC, 1990), or by enzymatic hydrolysis (Cook, 1978; Essers et al., 1993; O'Brien, Taylor & Poulter, 1991). The acid hydrolysis method involves hydrolysis of cyanogens in 2 M H_2SO_4 at 100 °C for 50 min, which is laborious and slow. Autolysis, the hydrolysis of linamarin by endogenous linamarase, is more suitable for fresh cassava material, but the reaction time can take up to 24 h for certain samples like sun-dried cassava flours (Bradbury & Bradbury, 1994). Another disadvantage of this method is that it cannot be applied to cooked or roasted products since the endogenous enzyme is permanently inactivated. In enzymatic hydrolysis, linamarase is added to the acid-extracted sample after pH adjustments (pH 5–6). Enzymatic breakdown of cyanogenic glycosides is rapid at around 30 °C and takes less than 15 min depending on enzymatic activity (Cooke, 1978; Essers et al., 1993; Haque & Bradbury, 1999; Nambisan, 1999). Linamarase can be easily isolated from cassava latex (Haque & Bradbury, 1999; Nambisan, 1999). Both acid and enzymatic hydrolysis methods convert linamarin to cyanohydrins, which further decompose to cyanide and acetone at alkaline pH levels (Bradbury & Bradbury, 1994).

Various methods have been developed to determine endogenous cyanide liberated from cyanogenic glycosides. In the titration method, endogenous cyanide is precipitated with $AgNO_3$ (AOAC, 1990) after steam distillation of hydrogen cyanide from autolysed cassava samples (AOAC, 1990). A disadvantage of this method is the loss of cyanide during the distillation process (Borges et al., 1993). The alkaline picrate method is a semi-quantitative method in which cyanide reacts with alkaline picrate paper, and the change of colour is matched against a colour chart, the resulting chromophore can also be dissolved from the picrate paper for more accurate quantitative determination using spectrophotometer (Bradbury, 2009; Bradbury, Egan, & Bradbury, 1999). Although the picrate method is easy to use, it has certain disadvantages. The reaction is very slow (~16 h), the chemical needs special handling and storage, and the response is sometimes imprecise. Cyanide detection by picrate was also used in a micro diffusion method, where the change of colour of picrate pre-coated in ion-exchange sheet is read in a reflectometer (Saka, Mhone, & Brimer, 1998). The method, which is based on the König reaction in which CN^- is oxidised to a cyanogen halide by chloramine T (Cook, 1978; Essers et al., 1993; O'Brien et al. 1991), is the most important and most accurate colorimetric method. The cyanogen halide reacts with pyridine or a related compound to produce a dialdehyde, which is then coupled with primary amines or compounds with active methylene groups such as pyrazolone or barbituric acid to yield a coloured complex (Lambert, Ramasamy, & Paukstelis, 1975). The following combination of reagents have been developed to produce colours: pyridine/pyrazolone (Cooke, 1978), pyridine/barbituric acid (Bradbury et al., 1991; Mendoza, Kojima, Iwatsuki, Fukuda, & Uritani, 1984), isonicotinic acid/barbituric acid (Nagashima, 1978) and isonicotinic acid/1,3-dimethylbarbituric acid (Essers et al., 1993; Meeussen, Temminghoff, Keizer, & Novozamsky, 1989). All these multi-step reactions are relatively complex and the methods can only be carried out by trained personnel. Most recent published work related to the determination of cyanide in cassava products have used the picrate sensor (Banea et al., 2012; Bradbury & Denton, 2010; Burns, Bradbury, Cavagnaro, & Gleadow, 2012; Burns, Gleadow et al., 2012), despite its above mentioned disadvantageous (Bradbury et al., 1999, 2009). This behaviour can be rationalised by the lack of a suitable alternative.

Consequently, development of new, straightforward and rapid tests for the detection of cyanide in cassava products is crucial. Recently, the development of metal-based chemosensors for detecting endogenous cyanide has attracted much attention and future applications in food safety control have been proposed. The aquacyanocobyrinic acid chemosensor (ACCA), a Co(III) metal complex (a derivate of vitamin B_{12}), seems to be advantageous compared to other colorimetric reagents since it reacts with cyanide within seconds and detection does not interfere with common anions or biological material, as demonstrated by Zelder (2008) and Männel-Croisé, Probst, and Zelder (2009). Furthermore, the chemicals are non-toxic and the system is easy to handle (Zelder, 2008).

In this study, we extend the first studies and demonstrate straightforward applicability of the corrin-based chemosensor for the instantaneous detection of endogenous cyanide in various cassava products such as fresh cassava roots, boiled fresh cassava roots and dried cassava roots, and describe a spectrophotometric method for the rapid quantification of total cyanogenic glycosides (CNp).

2. Materials and methods

2.1. Samples

All samples of cassava products were collected in Mozambique. Samples from fresh roots were harvested in two different forms: two roots from unnamed bitter cassava, identified as roots 1 and 2, and two roots from an unnamed sweet variety, identified as roots 3 and 4. The pulp and cortex of the fresh roots were used in the analysis. CNp were also measured in the boiled pulp of roots 3 and 4. Two samples of cassava flour from sun-dried bitter cassava roots and one sample of roasted shredded cassava roots (garri) were also used for CNp analysis.

2.2. Chemicals

The following chemicals were used: sodium hydroxide (Sigma–Aldrich, CAS 1310-73-2), potassium cyanide (Fluka Biochemika, CAS 151-50-8), orthophosphoric acid (Sigma–Aldrich CAS 7664-38-2), tri-sodium phosphate (Sigma–Aldrich CAS 7601-54-9), 1,3-dimethylbarbituric acid (Aldrich, CAS 769-42-6), isonicotinic acid (Aldrich, CAS 55-22-1), chloramine T (Sigma–Aldrich CAS 7080-50-4), glycine (Merck, CAS 56-40-6), α-amylase (Sigma–Aldrich CAS 9000-85-5) and Milli-Q water.

Aquacyanocobyrinic acid (ACCA) was synthesised as described elsewhere (Männel-Croisé & Zelder, 2009).

2.3. Chemical solutions

Stock solutions of NaOH (0.2 M), orthophosphoric acid (0.1 M), tri-sodium phosphate (0.1 M), phosphate buffer pH 6 (mixture of equal volume of phosphoric acid (0.1 M) and tri-sodium phosphate (0.1 M)) were prepared. Chloramine T was prepared freshly each day by dissolving (0.5 g) in water (25 ml). Glycine buffer (0.1 M) was prepared and the pH was adjusted to 9.5 with NaOH (1 M). For the standard curve, a potassium cyanide (300 μM) stock solution was prepared in NaOH (0.2 M; pH 12.4) or in glycine buffer (0.1 M, pH 9.5).

The stock solution of the aquacyanocobyrinic acid (ACCA) reagent was prepared by dissolving aquacyanocobyrinic acid (4 mg) in water (10 ml). The exact concentration of the ACCA sensor was calculated after diluting an aliquot (100 μl) with water (650 μl) followed by the full conversion of ACCA to DCCA with excess cyanide. From the absorbance of the diluted solution, $A_{366nm} = 1.015$ and the characteristic absorbance of dicyano-corrinoids at 366 nm $\varepsilon_{366nm} = 30400$ M^{-1} cm^{-1}, the molar concentration of ACCA in the stock solution was found to be 248 μM.

The isonicotinate/1,3-dimethylbarbiturate (ISO) reagent was prepared by adding NaOH (3.7 g), 1,3-dimethylbarbituric acid (7.0 g) and isonicotinic acid (5.7 g) to water (200 ml) and the pH was adjusted to a value between 7 and 8 with 1 M NaOH.

2.4. Preparation of latex extracts containing linamarase

The latex extracts containing linamarase used in the CNp enzymatic assays were prepared from the latex of cassava leaves as described by Haque and Bradbury (1999) and Nambisan (1999). Latex from about twenty cassava leaves was collected and added to distilled water (5 ml) in centrifuge tubes (10 ml). The mixture was shaken with a vortex mixer (Stuart) for 10 min and centrifuged at 3000 rev min^{-1} for 15 min. The supernatant enzyme solution was placed in plastic vials and stored at −20 °C until use. The enzymatic activity was determined by monitoring enzymatically released endogenous cyanide of cassava flour 1 as a function of time using the ACCA sensor. In this procedure, 50 µl of a 50-times diluted crude latex extract was incubated with 50 µl of cassava flour sample extract (identified as flour sample 1) as substrate sensor.

2.5. Preparation of cassava sample extracts

2.5.1. Extraction by homogenisation and centrifugation
Fresh cassava: a root was peeled, the pulp sliced twice longitudinally, and three slices were taken for analysis. Each piece of pulp (slice) was cut into smaller pieces and pounded, and the peel cortex was also cut into small pieces and pounded. A sample of pulp (20 g) or cortex (10 g) was then collected and added to either 0.1 M phosphoric acid (100 ml) or Milli-Q water (100 ml) in a beaker. Boiled cassava roots: a root was peeled and the pulp sliced twice longitudinally. Each slice of pulp was placed in a beaker with about 500 ml of water. It was then boiled for 5 min, removed from the water and crushed. A sample (20 g) was then added to either 0.1 M phosphoric acid (100 ml) or Milli-Q water (100 ml). Cassava flour or rale: samples were pounded and about 3 g of cassava flour and 12 g of rale were added to either 0.1 M phosphoric acid (100 ml) or Milli-Q water (100 ml). All the solutions were homogenised using an immersion blender. About 10 ml of each solution were placed in 10-ml centrifuge tubes and centrifuged for 15 min at 3000 rev min at 10 °C using x-15R centrifuge (Beckman Coulter Inc., California, USA). The supernatant was carefully removed via a pipette into plastic vials and stored at 4 °C until required for analysis. The rale sample contains gelatinized starch that swells in water forming a gel, so α-amylase (0.05 g) was added to the rale solution (100 ml) to liquefy the extract.

2.5.2. Extraction by pressing or direct addition to the water
Fresh cassava root pulp or cortex was crushed and pressed using a garlic press until 1 to 2 drops of juice were released into a beaker with Milli-Q water (5 ml). The drops were placed on a scale and the weight recorded. The solution was gently shaken by hand, and left to stand for 2–3 min before the incubation step. For cassava flour, about 2 mg was added to Milli-Q water (5 ml) and gently shaken by hand, and left to stand for 2–3 min before the incubation step.

For simplicity, samples extracted in water where the process involves homogenisation, centrifugation, pressing into water and direct dissolution of cassava flour into water are called 'water-extracted' in this study. Samples extracted in phosphoric acid are called 'acid-extracted'.

2.6. Preparation of KCN standard curves

The concentration of CNp using the ACCA sensor was determined from standard curves. For this, ACCA (248 µM) was titrated with a KCN stock solution (300 µM) at pH 9.5. For phosphate buffer, the standard curve was obtained from KCN dissolved in 0.2 M NaOH. Volumes of 0, 15, 30, 45, 60 and 100 µl of KCN stock solution were placed in test tubes. If required, the volume in each tube was made up to 100 µl with 0.2 M NaOH that is defined as the sample volume. This was followed by addition of 0.1 M phosphate buffer at pH 6 (300 µl). The pH of the system was adjusted to 9.5 by adding 0.2 M NaOH (50 µl), followed by water (100 µl) and ACCA sensor (100 µl) to make a total volume of 750 µl. For glycine buffer the standard curve was obtained from KCN dissolved in 0.1 M glycine by removing the same volumes as above via pipettes and transferring to the test tubes. The volume in each tube was made up to 100 µl with 0.1 M glycine buffer at pH 9.5. The total volume of the system was obtained by adding 0.1 M glycine buffer pH 9.5 (50 µl) to the test tubes, followed by water (500 µl) and ACCA sensor (100 µl).

Absorbance scanning measurements were carried out in the wavelength range between 300 and 650 nm using Varian Cary 50 Bio UV/visible spectrophotometer (Varian, Australia). The standard curves are referred to the concentration of KCN in the sample volume.

For both buffers, the pH of the system was kept around 9.5 ± 0.4, and the temperature of measurement was that of the room temperature, 22 °C.

2.7. Quantification of total cyanogenic glycosides in water- and acid-extracted samples

The procedure for both ISO and ACCA methods are summarised in the Table 1.

2.7.1. Quantification by aquacyanocobyrinic acid sensor
For quantification of CNp in the water- and acid-extracted samples, the extracts were first subjected to conditions whereby CNp was converted enzymatically to cyanohydrin (pH 5–6.5, 30 °C) and cyanohydrin to cyanide (pH 9.5). Finally cyanide was quantified spectrophotometrically using the ACCA chemosensor and the KCN standard curve. The steps of the procedure are sumarised in Table 1.

2.7.1.1. Samples extracted in water. The pH of the water-extracted samples was checked to ensure it was between 5.6 and 6.3. For the analysis, the water-extracted cassava samples (25–100 µl) and water (0–75 µl) were first placed in a stoppered test-tube, latex extract (50 µl) was added and the mixture incubated for 10 min at 30 °C in a water bath. After the incubation, 0.1 M of glycine buffer pH 9.5 (150 µl) and water (350 µl) were added. Finally ACCA sensor (100 µl) was added and the absorbance was recorded immediately. The total volume was 750 µl.

2.7.1.2. Samples extracted in phosphoric acid. The acid-extracted samples (25 to 100 µl) and water (0–75 µl) were placed in a stoppered test-tube, then 0.1 M phosphate buffer pH 6 (300 µl) and latex extract (50 µl) were added. The mixture was incubated for 10 min at 30 °C in a water bath. After the incubation, 0.2 M sodium hydroxide (200 µl) was added. Finally the absorbance was recorded after addition of ACCA sensor (100 µl).

For both water- and acid-extracted samples, the volume was corrected to a volume of 100 µl by the addition of water if required.

Measurements using glycine buffer have been only applied for water extracted samples and measurements using phosphate buffer only for acid-extracted samples. For simplicity, the former

Table 1
Proceedings of CNp determinations using ISO or ACCA sensors.

	ISO sensor	ACCA sensor	
Step 1	Extraction of cassava samples with H_3PO_4 0.1 M	Extraction of cassava samples with H_3PO_4 0.1 M	Extraction of cassava samples with H_2O
Step 2	Addition of phosphate buffer 0.1 M (pH 6)	Addition of phosphate buffer (pH 6)	Addition of linamarase for sundried, roasted and boiled samples (for fresh samples this step is not needed)
Step 3	Addition of linamarase for all samples	Addition of linamarase for all samples	Incubation (10 min)
Step 4	Incubation (10 min)	Incubation (10 min)	Addition of buffer (Glycine 0.1 M pH 9)
Step 5	Addition of NaOH 0.2 M	Addition of NaOH 0.2 M	Addition of ACCA sensor, no incubation is needed
Step 6	Addition of Buffer phosphate buffer 0.1 M (pH 6)	Addition of ACCA sensor, no incubation is needed	Absorbance reading at spectrophotometer
Step 7	Addition of Chloramine T 5%	Absorbance reading at spectrophotometer	
Step 8	Addition of ISO sensor		
Step 9	Incubation for 10 min		
Step 10	Absorbance reading at spectrophotometer		

measurement is abbreviated as ACCAwa and the latter as ACCAac, respectively.

For both water- and acid-extracted samples, the sample absorbance was corrected from sample background absorbance without ACCA sensor.

2.7.2. Validation, precision and accuracy

To evaluate the precision and accuracy, as well to validate the ACCA sensor, a known amount of KCN (30 µl of a 300 µM stock solution) was added to the following samples: cassava water extract, maize flour water extract and potatoe water extract. The cyanide concentration was determined using ISO and ACCA sensors. As a control. the sample extracts without added KCN were also measured. Each sample measurement was carried out in quintuplicate.

2.7.3. Determination by isonicotinate/1,3-dimethylbarbiturate sensor

Determinations of CNp with the ACCA chemosensor were compared to results using isonicotinate/1,3-dimethylbarbiturate (ISO) for samples extracted in phosphoric acid. The latter procedure has been described by Essers et al. (1993) and is summarized in Table 1.

2.8. Statistical analysis

Each sample of cassava was extracted three times. Determination of CNp of each extract was carried out at least three times. Statistical analysis of the data was carried out using MSTAT Software. Two-way ANOVA (analysis of variance) and Student's T linear regression analyses were performed.

3. Results and discussion

3.1. Calibration curves of aquacyanocobyrinic (ACCA) sensor in glycine (ACCAwa) and phosphate buffer (ACCAac)

Fig. 1a shows characteristic spectra of the titration of aquacyanocobyrinic acid with cyanide to dicyanocobyrinic acid, and this is accompanied by a redshift of the maxima. In particular, the increase of the wavelength at 578 nm at increasing cyanide concentrations indicates the formation of dicyanocobyrinic acid (DCCA). The shapes of the spectra are comparable for both buffer conditions. In Fig. 1b, the calibration curves at 578 nm at pH 9.5 are depicted after subtracting the absorbance of pure ACCA sensor. The calibration curves refer to a sample volume of 100 µl (for details see experimental part). With phosphate buffer, the sensitivity of the assay (0.015 AU/ppm) is slightly higher than the glycine buffer (0.014 AU/ppm). Linearity (R_2 > 0.999) is observed up to 200 uM in both buffers. The glycine buffer was used for samples that are extracted in water because it is cheaper than other buffers. Phosphate buffer was applied for samples that are extracted in phosphoric acid as described previously (Cooke, 1978; Essers et al., 1993).

3.2. Enzyme activity of cassava latex extract

For processed cassava products and phosphoric acid-extracted samples, an external enzyme, linamarase, is required for hydrolysis of residual cyanogenic glycoside (Cooke, 1978; Essers et al., 1993). This is of particular importance for the fast aquacyanocobyrinic acid
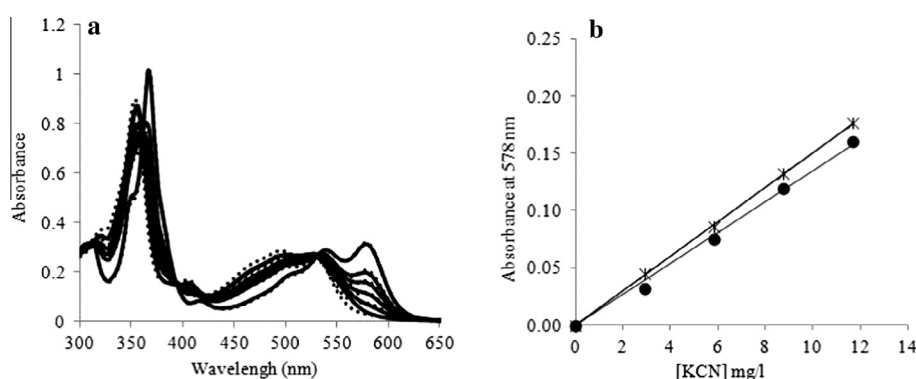

Fig. 1. (a) Absorbance spectra of aquacyanocobyrinic acid (33 µM) with increasing quantity of KCN. Solid line = glycine buffer, dashed line = phosphate buffer. (b) Standard curve of absorbance read at 578 nm against concentration of KCN in linear region (2.92 to 11.7 mg/l of KCN; for details see experimental part), glycine buffer (•) and phosphate buffer (*).

assay because of the sensor's high affinity and specificity to cyanide. Cyanide binding occurs extremely fast and the colour change under spectrophotometric conditions is instantaneous. Consequently, when determining instantaneous cyanogenic glycosides, the enzymatic conversion could be too slow at low enzyme concentrations.

The apparent latex-extracted enzyme kinetics was measured using the cassava flour sample and the aquacyanocobyrinic sensor at pH 9.5 (glycine buffer = 20 mM) at 22 °C as described earlier.

Using the results of absorbance change during incubation (Fig. 2), the time of the reaction was 26 min for the addition of 1 µl of crude extract to the 1 ml cuvette with 1 cm path so, by approximation, adding 50 µl of crude extract, the reaction could be complete in 1 min.

The latex crude extract containing the enzyme (centrifuged and not centrifuged) was free of CNp as checked by both sensors (ISO and ACCA). Of note, the non centrifuged crude extract containing the enzyme can be used directly without need of additional filtration.

3.3. Comparison of aquacyano-cobyrinic acid sensor in glycine buffer (ACCAwa) and phosphate buffer (ACCAac) with isonicotinate/1,3-dimethyl barbiturate (ISO) sensor

The detection of total CNp in cassava products using the enzymatic assay and the new ACCA method was compared to the quantification of CNp with isonicotinate/1,3-dimethyl barbiturate (ISO) as developed by Essers et al. (1993). ISO sensor was proposed as substitute for a sensor based on a toxic pyridine/pyrazolone solution developed by Cooke, 1978 and improved by O'Brien et al. (1991), which both require chloramine T. Typical sample
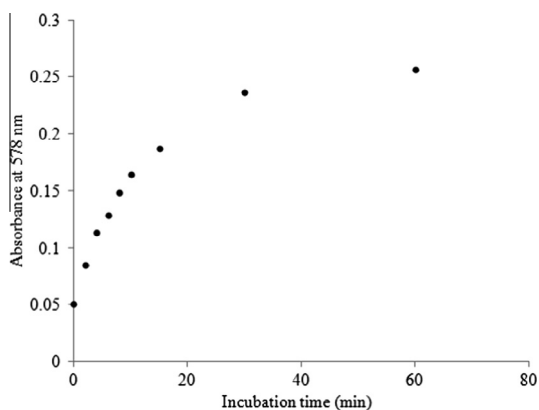
types – fresh and boiled cassava tissue, flour and rale spanning a range of enzymatic activities and cyanide contents – were used for the tests, as summarised in Table 2. The comparison of CNp values between the three methods was analysed though linear regression of CNp values from 27 extracts of 9 different samples, since each sample was extracted 3 times. Determination of cyanide in samples extracted for two days in phosphoric acid gave high correlation ($R^2 = 0.997$) and a slope of 0.946. A standard error (SE) of 0.015 is observed between ISO and the ACCAac sensor (Fig. 3a). Samples extracted in water were analysed immediately after the extraction with ACCAwa, showing high correlation with samples extracted in phosphoric acid ($R^2 = 0.997$; slope 1.00; SE = 0.016) and measured with ISO. The corresponding curve is shown in Fig. 3b. CNp values obtained by using the ACCAchemsoensor in
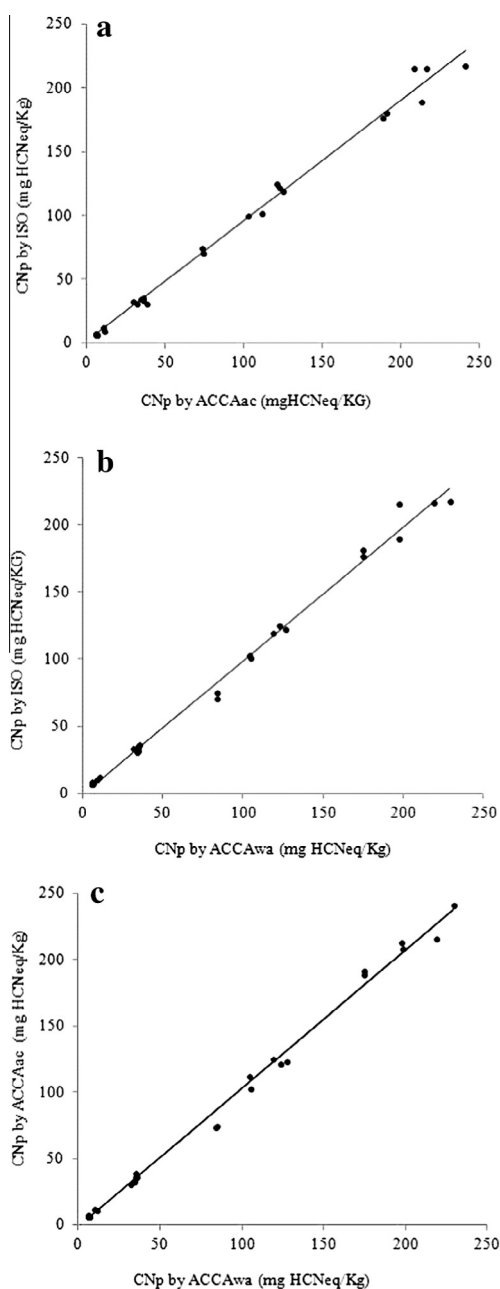

Fig. 2. Kinetic trace of absorbance reading of liberation of HCN during incubation of cassava flour 1 with diluted latex solution (50× dilution).

Fig. 3. Comparison of the three methods in terms of determination of total cyanogenic glycoside (CNp) by linear regression. In (a) correlation coefficient $R^2 = 0.997$, intercept = 1.34, slope = 0.946, standard error (SE) = 0.015. In (b) $R^2 = 0.997$, intercept = −0.69, slope = 1.00 SE = 0.016. In (c) $R^2 = 0.997$, intercept = 2.24, slope = 0.949, SE = 0.014.

Table 2
Average CNp (mg HCN equiv./kg) results obtained by the three methods. Uncertainty is the standard deviation of three independent sample extracts.

Cassava samples	ISO	ACCA acid	ACCA water
Cassava flour 1	220 ± 4	232 ± 10	224 ± 7
Cassava flour 2	122 ± 5	123 ± 2	123 ± 6
Pulp 1	100 ± 2	107 ± 4	104 ± 1
Pulp 2	73 ± 2	73 ± 1	84 ± 1
Cortex 1	186 ± 20	201 ± 16	197 ± 1
Cortex 2	181 ± 15	185 ± 9	174 ± 1
Pulp 3	35 ± 1	36 ± 1	36 ± 1
Pulp 4	7 ± 1	7 ± 1	7 ± 1
Boiled pulp 3	31 ± 2	31 ± 5	33 ± 2
Boiled pulp 4	6 ± 1	6 ± 1	6 ± 2
Garri	11 ± 1	ND	10 ± 1

ND–not determined.

samples extracted in water (ACCAwa) and samples extracted in phosphoric acid (ACCAac), also showed high correlation, $R^2 = 0.997$, slope = 0.949, SE = 0.014 (Fig. 3c). We also performed two-way ANOVA on results produced by the three methods and also on log-scale readings in order to take into account the uneven distribution of replicate error. There was no significant difference between the methods in either case ($P = 0.983$, $P = 0.998$), nor was there a significant interaction between sample and method ($P = 0.12$; $P = 0.09$). ACCA chemosensor is an excellent substitute of the complex ISO system in dictating cyanide in enzymatic assay of CNp in cassava. Moreover, it is easier to handle, requires less sample preparation and offers unprecedented rapid detection.

The aquacyanocobyrinic chemosensor can be used for cassava samples that are extracted in water, but the determination should be carried in same day which may limit the number of samples that can be easily handled. Samples extracted in water and stored for two weeks at 5 °C showed 50% lower result for fresh roots and 20% lower result for cassava flour. The high loss of cyanide in fresh root extract is due to the higher enzymatic activity than that in dried cassava flour. For samples extracted in phosphoric acid the cyanide content showed stability for 2 weeks, corresponding with results obtained by Essers et al. (1993).

Fig. 4 compares CNp measurements of different samples in the absence or presence of additional external enzyme. The results indicate that external enzyme needs to be added to cassava flour, boiled cassava roots and roasted cassava roots (rale) because endogenous enzymes were inactivated. In contrast, there is no need to add external enzyme for fresh roots. Partial inactivation of the enzyme is observed for water-extracted cassava flour. This behaviour is in agreement with observations by Bradbury (2006).

3.4. Validation, precision and accuracy

To validate and evaluate the precision and accuracy of the ACCA chemosensor, a defined amount of KCN was added to the following samples: cassava water extract, maize flour water extract and potatoes water extract. KCN concentrations were determined using ISO and ACCA chemosensors. The results are summarised in Table 3. Initially, the measurements were carried out in pure sample extracts. As expected, maize and potatoe extracts did not respond to both chemosensors confirming the absence of cyanide. In contrast, cassava extracts responded to both chemosensors. Moreover, both chemosensors responded comparably to the presence of additional exogenous KCN in the samples extracts. The standard deviation of ACCA is between 0.033 and 0.066 and that of ISO between 0.025 and 0.107. The percentage of error for the measurements with ACCA and ISO are between 4.1% and 7.3% for the former and 5.2% and 9.6% for the latter, respectively. The precision and accuracy of the measurements is therefore considered high and the percentage of error as still acceptable.
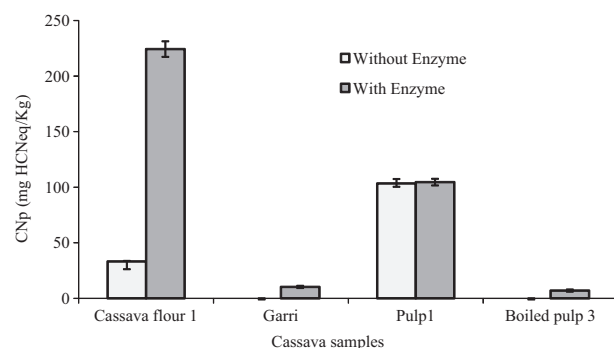

Fig. 4. Effect of addition of external linamarase on total cyanide detected by ACCAwa. The error bars show standard deviation of three independent sample extracts.

3.5. Direct measurement of samples

Additional experiments were carried out in fresh roots (pulp and cortex) and in cassava flour with the aim of accelerating the speed of cyanide determinations in cassava samples. Therefore it was tested whether the time consuming step of sample preparation including either acid or water extraction and centrifugation can be avoided. Cyanide determinations in drops of juice from the pulp resulted in no significant ($p > 0.05$) difference of cyanide concentrations whether they were extracted in water or phosphoric acid (see Fig. 5). This result appears of importance because pulp is the edible part of the root and represents also the main raw material for industrial cassava processing. Therefore rapid screening of CNp contents in pulp containing samples is proposed by this simplified squeezing method. However, this situation does not appear to be true for cortex samples (peel of internal roots) because the squeezing method gave significantly lower results. The reason is probably the higher content of fibre in cortex than in pulp. As a consequence the composition of the drop originates from rather extracellular than vacuolar liquid, whereas only the latter contains CNp. It is noteworthy that cortex is removed during the preparation of flour or boiled fresh roots. It has also been demonstrated that the sample preparation for CNp measurements in cassava flour can be simplified. Short extraction (2 min) of the sample with water (400 µg flour per ml of water) was sufficient to obtain comparable values for CNp than with the standard extraction method when using the ACCA chemosensor.

These results show the benefitial advantages of ACCA chemosensor compared to other reagent mixtures. The study demonstrates that the ACCA chemosensor can detect cyanide in various different cassava samples independent of the type of sample extraction. This behaviour makes quantification of CNp straightforward and rapid and applications in industrial screening can be

Table 3
Mean, standard deviation and percentage of error of data values of concentration calculated from absorbance reading and the standard curve using ISO or ACCA sensors.

| Samples	Cassava water extract (50 µl)		Cassava water extract (50 µl) + 300 µM KCN (30 µl)		Maize flour water extract (50 µl) + 300 µM KCN (30 µl)		Potatoes water extract (50 µl) + 300 µM KCN (30 µl)	
Sensor	ISO	ACCA	ISO	ACCA	ISO	ACCA	ISO	ACCA
Conc. reading (mg/l)	3.40	3.37	8.75	8.74	5.39	5.60	5.31	5.52
	3.40	3.45	8.75	8.74	5.39	5.60	5.39	5.44
	3.43	3.37	8.70	8.66	5.36	5.69	5.31	5.44
	3.40	3.37	8.75	8.74	5.36	5.60	5.34	5.44
	3.35	3.54	9.00	8.74	5.39	5.60	5.34	5.44
	3.40	3.42	8.79	8.73	5.38	5.62	5.34	5.45
Mean	3.40	3.42	8.79	8.73	5.38	5.62	5.34	5.45
STDV	0.025	0.066	0.107	0.033	0.012	0.033	0.028	0.033
Estimated true value	–	–	(3.40 + 5.85)	(3.42 + 5.85)	5.85	5.85	5.85	5.85
% Error			5.22	6.24	8.78	4.10	9.60	7.25
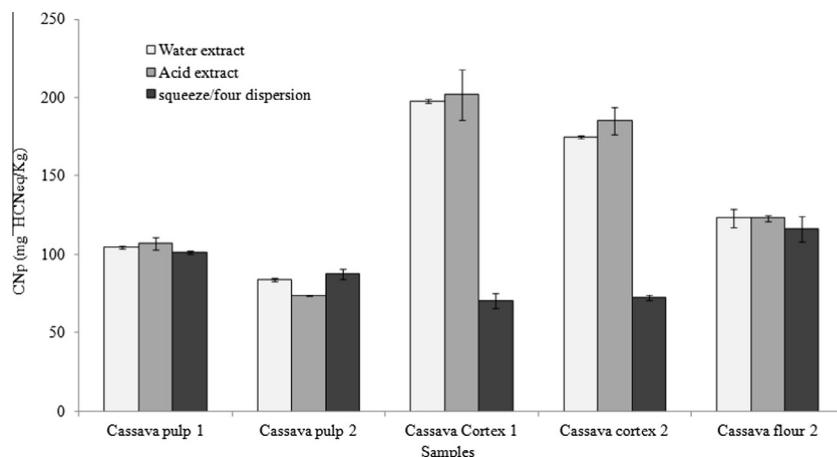
Fig. 5. Comparison of the result of three types of sample extraction on total detected cyanide from different samples of cassava using ACCA sensor. The error bars show standard deviation of three independent extracts.

envisaged. As described above, a group of ten fresh cassava root samples has been analysed in 20 min, including only 10 min for incubation for the enzymatic degradation of cyanogenic glycosides.

4. Conclusion

The determination of total cyanide in various cassava products using a aquacyanocobyrinic acid (ACCA) chemosensor is described. ACCA shows high precision and accuracy and the results are comparable to measurements with isonicotinate/1,3-dimethyl barbiturate (ISO). Application of ACCA for detecting total cyanogenic content in cassava products is straightforward and advantageous compared to ISO. The ACCA chemosensor is straightforward to use, has high affinity to cyanide, and the colour change is instantaneous. It was shown that the ACCA method can be applied for the instantaneous analyses of water-extracted samples as well as for monitoring cyanide in stored samples that were extracted with phosphoric acid. Cyanide can also be measured directly in a drop of fluid squeezed out from cassava pulp or in a water-cassava fluor mixture without any additional sample preparation. The enzymatic activity in the cassava leaf latex extract is sufficient to perform the assay on processed cassava.

References

AOAC. (1990). Hydrocyanic acid in beans, alkaline titration method. *Offcial methods of analysis* (15th ed.), p. 1213). Arlington, Va, USA: AOAC Inc..

Banea, J. P., Nahimana, G., Mandombi, C., Bradbury, J. H., Denton, I. C., & Kuwa, K. (2012). Control of konzo in DRC using the wetting method on cassava flour. *Food and Chemical Toxicology, 50*, 1517–1523.

Borges, M. F., Fukuda, W. M. G., & Caldas, R. C. (1993). Avaliação de três métodos para determinação de cianeto de mandioca. *Revista Brasileira de mandioca, 12*, 75–80.

Bradbury, J. H. (2006). Simple wetting method to reduce cyanogen content of cassava flour. *Journal of Food Composition and Analysis, 19*, 388–393.

Bradbury, J. H. (2009). Development of a sensitive picrate method to determine total cyanide and acetone cyanohydrin contents of gari from cassava. *Food Chemistry, 113*(4), 1329–1333.

Bradbury, J. H., Bradbury, M. G., & Egan, S. V. (1994). Compararison of methods of analysis of cyanogens in cassava. In M. Bokanga, A. J. A. Essers, N. Poulter, H. Rosling, & O. Tewe (Eds.). *Proceedings of the international workshop on cassava safety* (vol. 375, pp. 87–96). Ibadan, Nigeria: Acta Horticulturae.

Bradbury, J. H., & Denton, I. C. (2010). Rapid wetting method to reduce cyanogen content of cassava flour. *Food Chemistry, 121*, 591–594.

Bradbury, M. G., Egan, S. V., & Bradbury, J. H. (1999). Picrate paper kits for determination of total cyanogens in cassava roots and all forms of cyanogens in cassava products. *Journal of the Science of Food and Agriculture, 79*, 593–601.

Bradbury, J. H., Egan, S. V., & Lynch, M. J. (1991). Analysis of cyanide in cassava using acid hydrolysis of cyanogenic glycosides. *Journal of the Science of Food and Agriculture, 55*, 277–290.

Burns, A. E., Bradbury, J. H., Cavagnaro, T. R., & Gleadow, R. M. (2012a). Total cyanide content of cassava food products in Australia. *Journal of Food Composition and Analysis, 25*(1), 79–82.

Burns, A. E., Gleadow, R. M., Zacarias, A. M., Cuambe, C. E., Miller, R. E., & Cavagnaro, T. R. (2012b). Variations in the chemical composition of cassava (manihot esculenta crantz) leaves and roots as affected by genotypic and environmental variation. *Journal of Agricultural and Food Chemistry, 60*, 4946–4956.

Ciglenečki, I., Eyema, R., Kabanda, C., Taafo, F., Mekaoui, H., & Urbaniak, V. (2011). Konzo outbreak among refugees from Central African Republic in Eastern region, Cameroon. *Food and Chemical Toxicology, 49*, 579–582.

Cliff, J., Muquingue, H., Nhassico, D., Nzwalo, H., & Bradbury, J. H. (2011). Konzo and continuing cyanide intoxication from cassava in Mozambique. *Food and Chemical Toxicology, 49*, 631–635.

Conn, E. E. (1994). Cyanogenesis – a personal perspective. In M. Bokanga, A. J. A. Essers, N. Poulter, H. Rosling, & O. Tewe (Eds.). *Proceedings of the international workshop on cassava safet* (vol. 375, pp. 31–43). Ibadan, Nigeria: Acta Horticulturae.

Cooke, R. D. (1978). An enzymatic assay for the total cyanide content of cassava. *Journal of the Science of Food and Agriculture, 29*, 345–352.

Essers, A. A., Bosvel, M., van der Grift, R., & Voragen, A. (1993). Studies on the quantification of specific cyanogens in cassava products and introduction of a new chromogen. *Journal of the Science of Food and Agriculture, 63*, 287–296.

FAO. (2013). *Agricultural statistics*. Rome: Food and Agricultural Organization of the United Nations [http://www.faostat.fao.org, 03 January 2014].

Haque, M. R., & Bradbury, J. H. (1999). Preparation of linamarase solution from cassava latex for use in the cassava cyanide kit. *Food Chemistry, 67*, 305–309.

Lambert, J. L., Ramasamy, J., & Paukstelis, J. V. (1975). Stable reagents for the colorimetric determination of cyanide by modified König reactions. *Analytical Chemistry, 47*, 916–918.

Männel-Croisé, C., Probst, B., & Zelder, F. (2009). A straightforward method for the colorimetric detection of endogenous biological cyanide. *Analytical Chemistry, 81*, 9493–9498.

Männel-Croisé, C., & Zelder, F. (2009). Side chains of cobalt corrinoids control the sensitivity and selectivity in the colorimetric detection of cyanide. *Inorganic Chemistry, 48*, 1272–1274.

Meeussen, J. C. L., Temminghoff, E. J. M., Keizer, M. G., & Novozamsky, I. (1989). Spectrophotometric determination of total cyanide, iron-cyanide complexes, free cyanide and thiocyanate in water by a continuous-flow system. *Analyst, 114*, 959–963.

Mendoza, E. M. T., Kojima, M., Iwatsuki, N., Fukuda, H., & Uritani, I. (1984). Evaluation of some methods of analysis of cyanide in cassava. In I. Uritani & E. D. Reyes (Eds.), *Tropical root crops postharvest physiology and processing* (pp. 235–242). Tokyo: Japan Science Societies Press.

Mlingi, N. L. V., Nkya, S., Tatala, S. R., Rashid, S., & Bradbury, J. H. (2011). Recurrence of konzo in southern Tanzania: Rehabilitation and prevention using the wetting method. *Food and Chemical Toxicology, 49*, 673–677.

Nagashima, S. (1978). Spectrophotometric determination of cyanide with sodium isonicotinate and sodium barbiturate. *Analytica Chimica Acta, 99*, 197–201.

Nambisan, B. (1999). Cassava latex as a source of linamarase for determination of linamarin. *Journal of Agriculture and Food Chemistry, 47*, 372–373.

Nhassico, D., Muquingue, H., Cliff, J., Cumbana, A., & Bradbury, J. H. (2008). Rising African cassava production, diseases due to high cyanide intake and control measures. *Journal of the Science of Food and Agriculture, 88*, 2043–2049.

Nweke, F. I., Spencer, D. S. C., & Lynam, J. K. (2002). *The cassava transformation: Africa's best-kept secret*. USA: Michigan University Press.

O'Brien, G. M. O., Taylor, A. J., & Poulter, N. H. (1991). Improved enzymic assay for cyanogens in fresh and processed cassava. *Journal of the Science of Food and Agriculture, 56*, 277–289.

Rosling, H. (1988). *Cassava toxicity and food security – A review of health effects of cyanide exposure from cassava and of ways to prevent such effects*. Uppsala: International Child Health Unit, Uppsala University.

Saka, J. D. K., Mhone, A. R. K., & Brimer, L. (1998). An improved microdiffusion method with solid-phase detection for the determination of total cyanogens in fresh cassava. Comparison to the method of Cooke (1978). *Journal of the Science of Food and Agriculture, 76*, 334–340.

Westby, A. (2002). Cassava utilization, storage and small-scale processing. In R. J. Hillocks, J. M. Thresh, & A. C. Bellotti (Eds.), *Cassava biology, production and utilization* (pp. 281–300). Wallingford, UK: CABI Publishing.

Zelder, F. H. (2008). Specific colorimetric detection of cyanide triggered by a conformational switch in vitamin B12. *Inorganic Chemistry, 47*, 1264–1266.